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Quantitative fine-structural analysis of olfactory cortical synapses

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ABSTRACT **To determine the extent to which hippocampal synapses are typical of those found in other cortical regions, we have carried out a quantitative analysis of olfactory cortical excitatory synapses, reconstructed from serial electron micrograph sections of mouse brain, and have compared these new observations with previously obtained data from hippocampus. Both superficial and deep layer I olfactory cortical synapses were studied. Although individual synapses in each of the areas—CA1 hippocampus, olfactory cortical layer Ia, olfactory cortical area Ib—might plausibly have been found in any of the other areas, the average characteristics of the three synapse populations are distinct. Olfactory cortical synapses in both layers are, on average, about 2.5 times larger than their hippocampal counterparts. The layer Ia olfactory cortical synapses have fewer synaptic vesicles than do the layer Ib synapses, but the absolute number of vesicles docked to the active zone in the layer Ia olfactory cortical synapses is about equal to the docked vesicle number in the smaller hippocampal synapses. As would be predicted from studies on hippocampus that relate paired-pulse facilitation to the number of docked vesicles, the synapses in layer 1a exhibit facilitation, whereas the ones in layer 1b do not. Although hippocampal synapses provide as a good model system for central synapses in general, we conclude that significant differences in the average structure of synapses from one cortical region to another exist, and this means that generalizations based on a single synapse type must be made with caution.**

Because of its simple structure, the hippocampus has played the role for central synaptic physiology that traditionally was filled for peripheral synapses by the neuromuscular junction: much of what we know about central excitatory synapses comes from the study of monosynaptic connections between hippocampal regions CA3 and CA1. The structure of these same synapses has been most extensively studied quantitatively with reconstructions derived from serial sections [postsynaptic properties (1–3); presynaptic properties (4–6)]. To what extent is the structure of hippocampal synapses quantitatively like those found in other cortical regions? To approach this question, we have undertaken a quantitative analysis of excitatory synapses in another simple region, the piriform cortex. This area of cortex was selected for two reasons. First, it has a particularly simple structure and is suitable for the physiological analysis of cortical synaptic function; and second, it provides anatomically segregated synapses from two distinct sources—and with differing physiological properties—that can be compared.

The piriform (olfactory) cortex is the first cortical destination for olfactory information (7, 8). Olfactory cortex is an ancient structure—classified as paleocortex—found in all vertebrates and is supplied by its own "thalamus," the olfactory bulb, which provides a direct relay from the olfactory receptor surface. A particularly attractive feature of olfactory cortex is the simplicity of its structure (9–11). It can be divided into just three layers (I–III) and exhibits only a few morphologically distinct cell types [ref. 12; and see Stevens (13) for a comparison of descriptive terminologies]. Layer I consists of dendrites ascending from the underlying pyramidal (and granule) cells together with axons from main two sources: (*i*) the olfactory bulb and (*ii*) local feedback from olfactory cortical pyramidal cells. Layer I is subdivided into two distinct parts, with the superficial layer 1a containing the bulbar input axons and the deep layer 1b made up of recurrent excitatory axons from the cortex itself (11, 14–18). The intracortical associational fibers show a topographic distribution: the rostral piriform cortex projects to superficial part of layer 1b and the caudal piriform cortex to deeper layer 1b (19, 20).

Layer II contains most of the neuronal cell bodies in piriform cortex (9, 13, 14), and these are packed very densely, more densely than in any other cortical region except perhaps hippocampus. The most superficial neurons of layer II are granule cells, much like those in dentate, that lack basal dendrites. The deeper cells of layer II tend to have pyramidal-shaped cell bodies and basal dendrites, although the granule and pyramidal cell types are intermixed. The layer II cells send recurrent axons mainly rostrocaudally in piriform cortex that synapse on basal dendrites in layer III and then ascend to run in layer 1b, where they make many excitatory connections with apical dendrites of the underlying cells (18).

Layer III is more sparsely populated with pyramidal and polymorphic cells (7–9, 12, 14). Neurons in layer III have no apical dendrite and dendritic arborizations remain in layer III (9, 12). Layer III neurons send predominantly association fibers rostrocaudally in the piriform cortex (18).

We concentrated our attention on the excitatory synapses of layer 1a (from olfactory bulb) and layer 1b (recurrent from piriform cortex itself). These two segregated pathways not only originate in different locations but also exhibit different physiological properties: the axons from the olfactory bulb that project specifically to layer 1a show paired-pulse facilitation, whereas excitatory synapses from recurrent cortical fibers that arborize in layer 1b do not (21). We find that the general properties of the synapses in 1a and 1b overlap, but both are, on average, about 2.5 times larger than the comparable excitatory synapses found in hippocampus. Olfactory cortex synapses show tight correlations between the number of docked vesicles, active zone size, postsynaptic density size, and bouton volume, as do hippocampal synapses. The major differences we have found between olfactory synapses at the two locations (layer 1a vs. 1b) are the density of docked vesicles and the total density of vesicles in the bouton: layer 1a synapses have a density of 1,600 vesicles per μm^2 of active zone and a total vesicle density of 927 per μm^3, whereas the 1b synapses have higher densities: 2,600 vesicles per μm^2 of active zone and a total density of 3,192 vesicles per μm^3 of bouton axoplasm. Thus, the synapses in olfactory cortex differ significantly

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between the layers (more vesicles per synapse for the intracortical synapses in 1b) and also have structural properties that distinguish them from hippocampal synapses (they are larger).

MATERIALS AND METHODS

One adult mouse (7 months old) was perfused through the heart under deep Pentobarbitol (Abbott) anesthesia (4 mg/25 gm body weight). A short perfusion with oxygenated saline was followed by perfusion with 4% glutaraldehyde in 100 mM phosphate buffer (pH 7.3) at room temperature. The brain was dissected and immersed in the same fixative for 20 hr at room temperature. Vibratome sections (200 μm), including the piriform cortex, were cut and postfixed in 1% OsO_4 plus 1.5% K^+-ferrocyanide in 100 mM phosphate buffer (pH 7.3) at 4°C for 1 hr. Sections were contrasted en bloc in 2% uranyl acetate (aqueous) at 4°C for 1 hr. After dehydration in ethanol, the sections were flat-embedded in Epon. Specimens were trimmed to blocks, including the piriform cortex, and cut serially at silver. One series of micrographs from the superficial layer (1a) and one from the center of the deep layer (1b) were photographed at a magnification at ×14,000 from 32 consecutive sections. Micrographs were analyzed as described (5). Our analysis included all asymmetric synapses with round vesicles that were fully contained in the serial sections.

Terminology. We use the term "synapse" in a morphological sense: it is taken to include an active zone with docked vesicles and the associated postsynaptic density (this unit is sometimes called "the synaptic junction"). According to this terminology, a single bouton with three distinct active zones contacting three postsynaptic targets (spines or shaft) forms three synapses.

RESULTS

General Observations. In their general structure, excitatory synapses in olfactory cortex are like excitatory synapses elsewhere: they exhibit an active zone presynaptically (to which synaptic vesicles are docked) and a matching postsynaptic density. Most are made on spines. The presynaptic bouton is filled with a large number of synaptic vesicles; the synaptic cleft is quite uniformly 18–20 nm wide. In Fig. 1*A* we illustrate a typical synapse from layer 1a, and in Fig. 1*B* we show synapses from layer 1b. Note the lower densities of both docked and reserve vesicles in the 1a synapses as described in more detail below.

Presynaptic Characteristics. Olfactory cortical boutons most commonly make only a single synapse (80%), although two or more synapses per bouton are more common than in hippocampus (a summary quantitative comparison of olfactory and hippocampal synapses appears in Table 1). This is especially true of the 1b synapses, where one occasionally (4% = 1/28) even finds four synapses per bouton. Shaft synapses are less rare in olfactory cortex than in hippocampus with a prevalence of 16% for layer 1a synapses and 6% for layer 1b.

The mean active zone area of olfactory cortical synapses is the same for layers 1a and 1b, with a value of 0.095 μm^2 for both. Furthermore, the distribution of active zone areas is sharply skewed (the coefficient of variation = 1.1 for layer 1a and 0.9 for layer 1b), but has the same form for 1a and 1b synapses, as seen in the cumulative histograms presented in Fig. 2*A*; these two histograms are not significantly different (Kolmogorov–Smirnov test, $P > 0.10$). The number of docked vesicles per bouton is also skewed with a significant difference evident between 1a and 1b synapses. The density of docked vesicles for the synapses in layer 1a averages 1,600 μm^2 and is 2,600 μm^2 for the synapses in layer 1b (a significant difference—Kolmogorov–Smirnov test, $P < 0.001$; Fig. 2*B*). Docked vesicle densities (number of vesicles/μm^2 of active zone) are constant within a specific layer (coefficient of variation = 0.25 in layer 1a and 0.19 in layer 1b). Docked vesicle densities are independent of active zone size (Fig. 2*C*), but are different for the two synapse populations.

Olfactory cortical bouton volumes are 4–7 times larger than their counterparts in hippocampal region CA1 (Table 1) and are quite variable (Fig. 3*A*), with mean values of 0.367 μm^3 for 1a synapses and 0.208 μm^3 for 1b synapses. The bouton volume is positively correlated with active zone size ($r = 0.78$ for layer

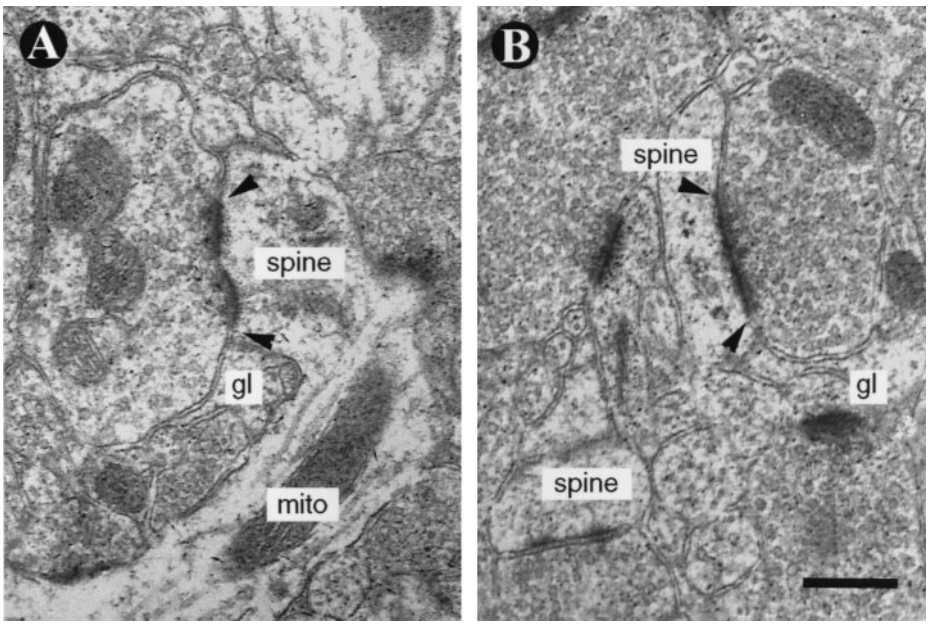

FIG. 1. Electron micrographs of synapses in layers 1a and 1b in mouse piriform cortex. Synapses show a clearly definable postsynaptic density (arrowheads), active zone, docked vesicles, and a large number of vesicles within the presynaptic bouton. [For a detailed description of the general morphology see Haberly and Feig (10)]. (*A*) Example of a synapse between a spine (spine) and a bouton in layer 1a. The synapse is perforated and the spine contains a spine apparatus. Synapses are very often encapsulated by glial processes (gl). (*B*) Example of synapses in layer 1b. Qualitatively the morphology is similar to layer 1a, but note the higher vesicle densities in layer 1b boutons as compared with layer 1a. Boutons and dendrites contain mitochondria (mito). (Bar = 0.5 μm.)

Table 1. Comparison of all quantitative synaptic data from piriform cortex and hippocampus

	Hippocampus	Piriform cortex	
Variable	CA1	Layer 1a	Layer 1b
Active zone	$0.039 \pm 0.022\ \mu m^2$	$0.095 \pm 0.101\ \mu m^2$	$0.097 \pm 0.087\ \mu m^2$
Postsynaptic density	$0.043 \pm 0.031\ \mu m^2$	$0.096 \pm 0.105\ \mu m^2$	$0.100 \pm 0.087\ \mu m^2$
Docked vesicle number	10.3 ± 5.6	16.4 ± 17.1	26.8 ± 23.8
Area per docked vesicle	62×62 nm	79×79 nm	62×62 nm
Bouton volume	$0.086 \pm 0.049\ \mu m^3$	$0.367 \pm 0.378\ \mu m^3$	$0.208 \pm 0.137\ \mu m^3$
Total number of vesicles	270 ± 176	354 ± 436.5	656 ± 423.7
Spine volume	$0.038 \pm 0.036\ \mu m^3$	$0.129 \pm 0.174\ \mu m^3$	$0.071 \pm 0.073\ \mu m^3$
Total synaptic vesicle density	$3{,}139/\mu m^3$	$927 \pm 446/\mu m^3$	$3{,}192 \pm 889/\mu m^3$
One synapse per bouton	90% (64)	81% (22)	82% (23)
Two synapses per bouton	8% (6)	19% (5)	11% (3)
Three synapses per bouton	2% (1)	0	4% (1)
Four synapses per bouton	0	0	4% (1)
Number of shaft synapses		16% (6)	6% (2)
Two synapses per spine	0	3% (1)	3% (1)
Two active zones per synapse	0	3% (1)	6% (2)
Total number boutons	71	27	28
Total number synapses	79	38	36

The first eight rows list the average values and the corresponding standard deviations for indicated quantities. Remaining rows give the percentage for different types of synapses (with the numbers in parentheses).

1a and $r = 0.71$ for layer 1b; Fig. 3*B*). The total vesicle pool within a bouton differs by about twofold between layer 1a and 1b (354 ± 436 for 1a vs. 656 ± 423 for 1b). The significantly larger total vesicle pool in layer 1b, together with the small difference in average bouton volumes (the 1b synapses are a little smaller), results in a threefold difference in vesicle density (number of vesicles/μm^2 of bouton) between the layers (Fig. 3*C*; Kolmogorov–Smirnov test, $P < 0.001$).

Postsynaptic Structure. Like active zone sizes, the areas of postsynaptic densities are broadly distributed with a marked skew (Fig. 4). As in hippocampus, the postsynaptic density closely matches the active zone in size and shape; therefore, the distributions of postsynaptic density sizes are just the same as the distributions of active zone sizes (Fig. 2*A*). Spine head volumes average 0.129 μm^3 for 1a synapses and 0.071 μm^3 for 1b synapses and are broadly distributed (Fig. 4) with a marked skew. Spine head volume is correlated with the active zone and postsynaptic density area (Fig. 4); therefore, large spines bear large synapses.

Relationships. The various structural characteristics of olfactory synapses are, as for hippocampus, highly correlated so that in general a large bouton has a large active zone opposite a large postsynaptic density situated in a large spine head. Furthermore, docked vesicle numbers are very tightly correlated with active zone area. A matrix of these correlations is provided in Fig. 4, where numeric values for correlations and scatter plots are shown.

DISCUSSION

Our results confirm previous observations on the ultrastructure of synapses within layer 1 of the piriform cortex (10, 11, 22, 23). We used three-dimensional reconstructions to obtain quantitative data on these synapses, however, whereas earlier investigators made measurements from single electron micrographs. One consistent result is that the lateral olfactory tract synapses in layer 1b have approximately a 3 times higher vesicle density ($\approx 3{,}000\ 1/\mu m^3$) than synapses in layer 1a ($\approx 1{,}000\ 1/\mu m^3$). In addition, here we show that the docked synaptic vesicle density is also significantly smaller in layer 1a than in 1b.

Comparison of Cortical Synapses. A comparison of our present observations with those on hippocampus (1, 5, 24) shows that hippocampal synapses are not, as a population, typical of all cortical synapses. Table 1 compares the average structural characteristics of excitatory synapses in CA1 of hippocampus and in layers 1a and 1b of piriform cortex. Because of the broad distributions of all variables we have measured, the synapses of all three types overlap to a great degree. One can easily find synapses in any of the three regions that are indistinguishable from those in other regions. Statistically, however, the three synapse populations are distinct. Olfactory cortical synapses are about 2.5 times larger than those in CA1 and exhibit more active zones per synapse than do their hippocampal counterparts. As noted by Yeow and Peterson (25) active zones rarely exceed an area of 0.4 μm^2, and larger synapses form multiple active zones per synapse rather than exceed this "limit." Olfactory synapses seem to fit this size principle: in our sample, only one synapse has an active zone area ($= 0.42\ \mu m^2$) that exceeds the 0.4 μm^2 demarcation line. Furthermore, shaft synapses are more common in olfactory cortex than in hippocampus, but are rather rare in both.

The densities of synaptic vesicles within a bouton are similar in layer 1b, in hippocampal region CA1 (Table 1; see ref. 5), and in mossy fiber terminals of the CA3 region (26), whereas boutons in layer 1a have lower densities. Similar low vesicle densities have been reported for the spinal cord [in cat $1{,}400\ 1/\mu m^3$ (27); in turtle $\approx 1{,}200\ 1/\mu m^3$ (25)]. Functional implications of the varying vesicle densities in various regions of the central nervous system are unknown, but presumably high densities reflect a higher overall release rate.

The 1a synapses have a lower density of docked vesicles than do the 1b synapses. The docked synaptic vesicle density is the same for 1b synapses and those in hippocampus, but the total number of vesicles docked at 1b synapses is, of course, larger because the active zones are larger. Interestingly, even though the 1a synapses also have larger active zones than the hippocampal synapses, the density of docked vesicles at the 1a synapses is sufficiently lower that the actual number of docked vesicles per synapse is about the same for layer 1a as it is for hippocampus.

Functional Implications. Because synapse structure is reflected in function, one would expect that these three populations of synapses would have distinct physiological properties. Synapses in layer 1a have long been known to facilitate, whereas those in 1b do not (21). The initial comparison of

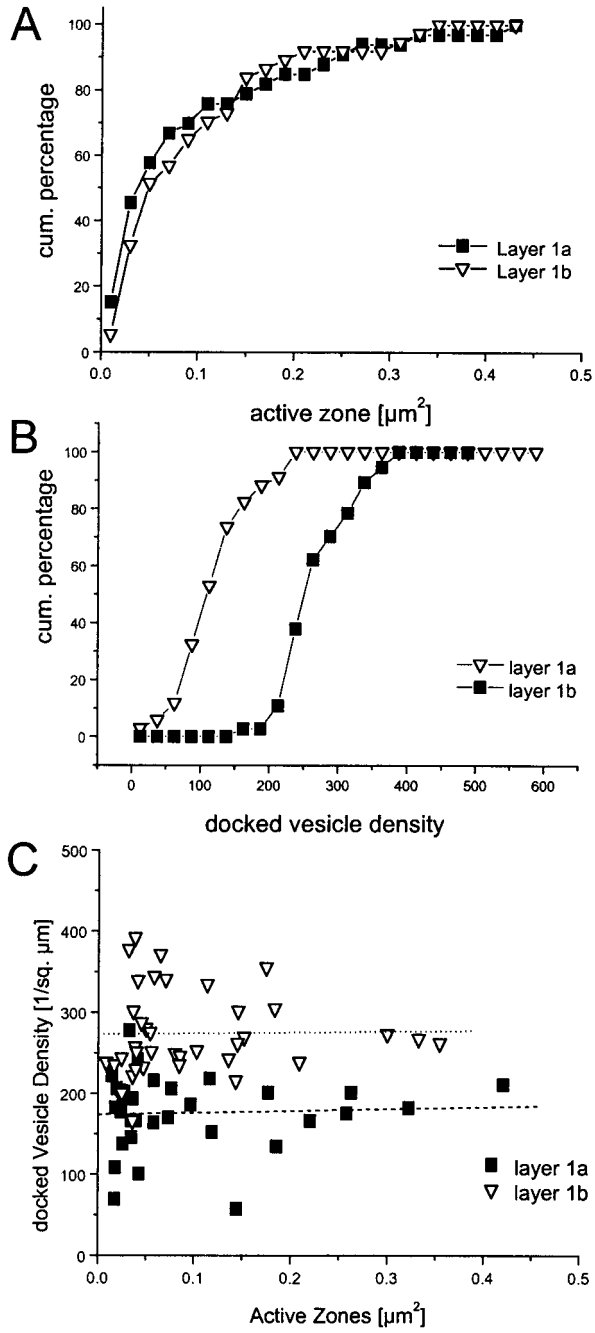

FIG. 2. Distribution of active zone areas and docked vesicles densities. (*A*) Cumulative histogram of active zone areas in layers 1a and 1b. Distributions are not significantly different (Kolmogorov–Smirnov test, $P < 0.1$). (*B*) Cumulative histogram of docked vesicle densities (number of vesicles/μm^2 of active zone) layers 1a and 1b. The layer 1a–1b densities are significantly different (Kolmogorov–Smirnov test, $P < 0.001$). (*C*) Docked vesicle densities as a function of active zone area. The parameters appear to be independent of each other ($r = 0.03$ and $r = 0.025$ for layers 1a and 1b, respectively).

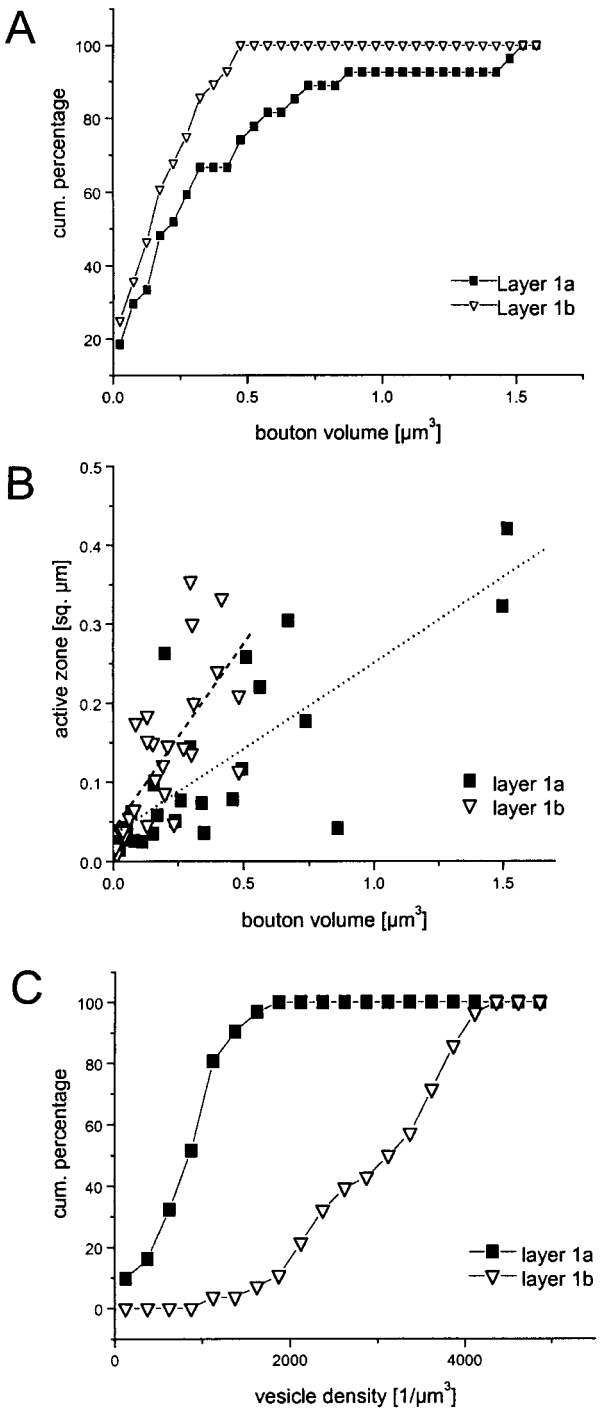

FIG. 3. Comparison of the presynaptic bouton volumes in piriform cortex. (*A*) Cumulative histogram of bouton volume in layers 1a and 1b. (*B*) Active zone size as a function of bouton volume. Although the volume is on average different for the two layers, the active zone area is highly correlated with the bouton volume in both layers ($r = 0.79$ and $r = 0.71$ for layers 1a and 1b, respectively). (*C*) Cumulative histogram of total synaptic vesicle densities for layers 1a and 1b (number of vesicles/μm^3 of bouton).

functional properties with structure (21) seemed to show a correlation with the vesicle density in the bouton. Synapses in layer 1b have a high vesicle density and do not show paired-pulse facilitation, whereas layer 1a synapses have a low vesicle density and facilitate. This correlation and the fact that paired-pulse facilitation depends on calcium (28) led Bower and Haberly (21) to hypothesize that the high vesicle density buffers calcium more effectively and thus prevents facilitation. A comparison with synapses in CA1 and mossy fiber terminals in CA3, however, reveals that these special hippocampal synapses also have high vesicle densities, equal to those in layer 1b (Table 1), but do show facilitation (29, 30). Similarly, group 1a boutons within the spinal cord have a vesicle density comparable to layer 1a olfactory synapses, but exhibit little or no facilitation (31). Altogether then presence or absence of facilitation does not seem to be explained by vesicle density.

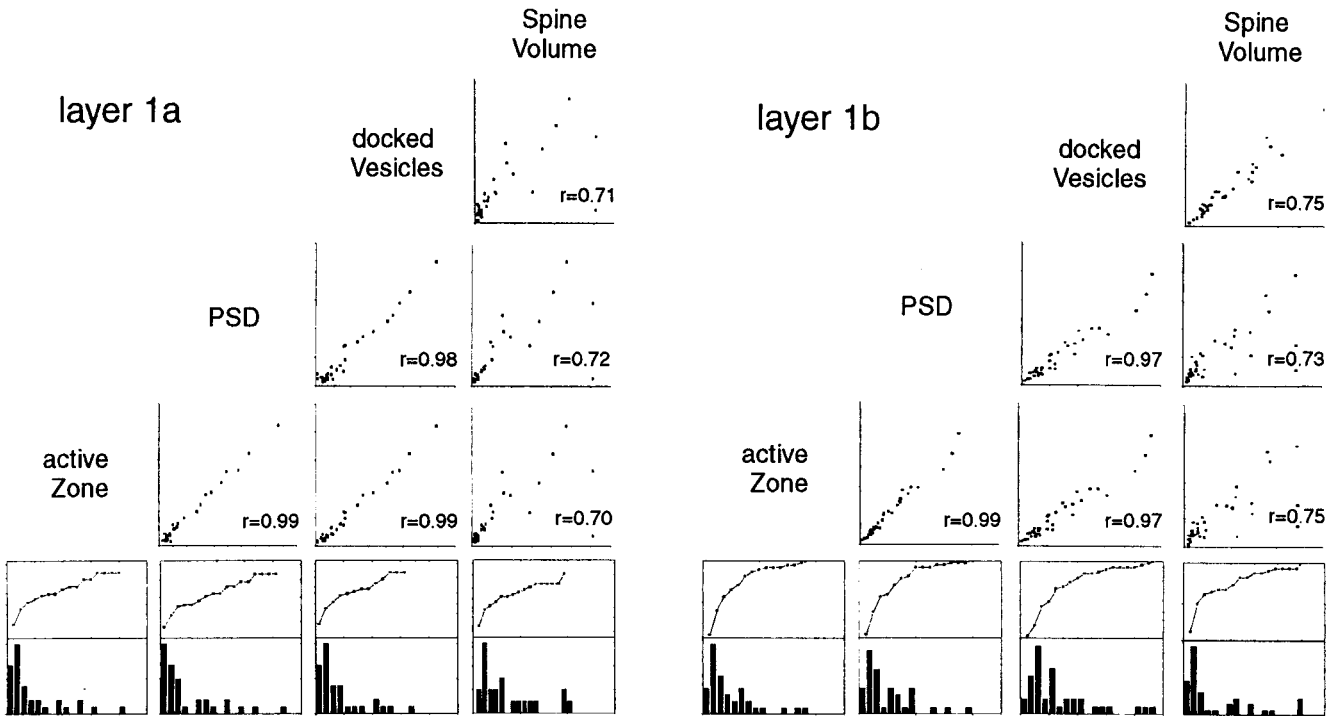

FIG. 4. This figure, arranged like the part of a 4×4 matrix below the diagonal, exhibits correlations between the various measured quantities (in layer 1a on the left and layer 1b on the right): active zone size, postsynaptic density size (PSD), number of docked vesicles, and spine head volume. Each of the graphs in this array is a scatter plot of the indicated variables with the associated correlation coefficient exhibited. For example, the scatter plot of postsynaptic density area as a function of spine volume for layer 1b synapses is the far right graph in the second row from the top; the correlation coefficient for these two variables is $r = 0.73$. To indicate the shape of the histograms that specify the relative frequency of the different variables we have included the bottom row in the graph that is subdivided into two with the bottom graph being the histogram for the variable indicated at the top of the column and the upper graph the corresponding cumulative histogram. For example, the left-most pair of bottom row graphs shows the distribution of active zone areas for layer 1a synapses and the fifth graph in the bottom row is the distribution of active zone areas for the layer 1b synapses; the cumulative histograms (top half of the row) also appear in Fig. 2*A*. Because the variables are all so highly correlated, all of the histograms have the same general shape. For the average values and standard deviations of the various variables see Table 1.

Studies (30, 32) have demonstrated that, at hippocampal synapses, the amount of facilitation is related to the number of docked vesicles: larger numbers of docked vesicles are associated with less paired-pulse facilitation. The mechanism proposed by Dobrunz and Stevens (30) should apply to olfactory synapses as well, and predicts that layer 1a and 1b synapses should differ in their facilitation. The size of the docked vesicle pool in layer 1a is not significantly larger than that of the hippocampal synapses. All else equal, we would thus predict that the facilitation of the layer 1a synapses would be about the same as for hippocampal synapses, and this is indeed what is observed. The layer 1b synapses do have a significantly larger docked vesicle pool and so they would be expected to exhibit less facilitation than the hippocampal synapses, as they do. Our observed structural differences between the three populations of synapses (CA1, 1a, and 1b) thus can account for the similarities and differences across synapse types of a physiological characteristic, facilitation. In addition, Pierce and Mendell (27) found a large pool of docked vesicles at active zones in group 1a fibers of the spinal cord and those synapses showed no or only a small amount of paired-pulse facilitation as noted earlier (31, 33).

Although the hippocampus provides a good model for the study of central synapses, one must be cautious in making generalizations from properties of hippocampal synapses to those in other regions. From our structural study, we expect that properties of synapses in different regions will overlap, but we can also anticipate that the average properties of a population can be quite different. It remains for future investigations to define the range of structural and functional characteristics found through the various brain areas.

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